Pestic. Sci. 1996, **46**, 267–275

Glutathione Transferase Activities and Herbicide Selectivity in Maize and Associated Weed Species*

Pamela J. Hatton,[a] David Dixon,[a] David J. Cole[b] & Robert Edwards[a]‡

[a] Department of Biological Sciences, University of Durham, Durham DH1 3LE, UK
[b] Rhône-Poulenc Agriculture Ltd, Ongar, Essex CM5 0HW, UK

(Received 20 May 1995; revised version received 24 June 1995; accepted 22 September 1995)

Abstract: The role of glutathione transferases (GSTs) in the selectivity of the herbicides alachlor, atrazine, fluorodifen and metolachlor, which are detoxified by glutathione conjugation in plants, was determined in seedlings of maize (*Zea mays* L.) and the associated weed species *Abutilon theophrasti* Medic., *Digitaria sanguinalis* (L.) Scop. *Echinochloa crus-galli* (L.) Beauv., *Panicum miliaceum* (L.), *Setaria faberi* Herrm. and *Sorghum bicolor* (L.) Moench. The availability of glutathione was also determined in all species and tissue concentrations were found to be in the range 120–160 μM for all species except *D. sanguinalis* and *S. bicolor*, which contained half this amount. GST activities toward the herbicides were determined in crude protein extracts from the plants using HPLC to quantify the biosynthesis of the herbicide conjugates. The specific activities of the GSTs toward the substrates were in the order alachlor > fluorodifen > atrazine > metolachlor in all species except *A. theophrasti*, where fluorodifen was a better substrate than alachlor. In most cases there was a good correlation between GST activities and the selectivity of the herbicides applied pre-emergence. In the case of atrazine, GST activities were also related to the relative rates of herbicide conjugation *in vivo*. In contrast, there was no simple relationship between glutathione availability and the selectivity of the herbicides. However, with alachlor there was evidence that glutathione availability was limiting GST activity and influencing tolerance.

Key words: glutathione transferases, herbicide detoxification, selectivity.

1 INTRODUCTION

The relative rate of herbicide detoxification between tolerant crops and susceptible weed species is frequently cited as a major determinant in herbicide selectivity.[1] A well-established example is the correlation between the relative rates of detoxification of the chloro-*s*-triazine herbicide atrazine by glutathione conjugation in susceptible and tolerant crops. Maize plants (*Zea mays* L.) rapidly detoxify atrazine by glutathione conjugation and are tolerant to this herbicide, while susceptible species such as pea metabolise atrazine more slowly by *N*-dealkylation and are susceptible.[2] The conjugation of atrazine with glutathione in maize is catalysed by glutathione transferases (GSTs),[3] with cultivars containing high levels of this specific GST activity being more tolerant than plants with low levels.[4] The GSTs in maize with activity towards herbicides have been studied more than in any other plant and it is now known that, in addition to the GSTs with activity toward atrazine,[3] there are at least four other GSTs, termed GSTs I–IV which can detoxify the chloroacetanilide herbicides metolachlor and alachlor.[5,6] GSTs I and III are expressed constitutively, while GSTs II and IV accumulate following treatments with herbicide safeners.[3,5,6] In

* Based on a paper presented at 'Research into Bioactive Molecules', a symposium for Postgraduate Scientists organised by T. Joseph-Horne on behalf of the Physicochemical and Biophysical Panel of the SCI Pesticides Group and held at the Frythe, Welwyn Garden City, Herts., UK, on 15 March, 1995

‡ To whom correspondence should be addressed.

Pestic. Sci. 0031-613X/96/$09.00

maize, there is also some evidence to suggest that highly reactive herbicides, or their metabolites, such as the sulfoxide of EPTC, may be detoxified following their spontaneous conjugation with the relatively high concentrations of glutathione present in this species.[7]

From these observations it can be concluded that GSTs and glutathione availability play an important role in herbicide tolerance in maize. However, the importance of this detoxification system in other plants is less well defined, as, although GSTs, or genes encoding GSTs, have been identified in a wide range of plant species, their role in herbicide conjugation has only been determined in a handful of cases. Thus, GSTs have been partially characterised in pea[8] and spruce[9] with activity toward diphenylether herbicides, in sorghum with activity toward metolachlor,[10] and in the weed species *Abutilon theophrasti* Medic.[11] and *Panicum miliaceum* (L.)[12] with activity toward atrazine. Recently, GST activities toward atrazine and metolachlor have been reported in a number of *Setaria* species[13] but, with a few exceptions, there has been little focus on the significance of GSTs in the responses of weed species to herbicides.

We are interested in identifying the range of GST activities toward herbicides in major crop species and their associated weeds and in critically determining the role of these enzymes in herbicide selectivity. To these ends, an assay has been developed which is suitable for determining GST activities toward a variety of herbicide substrates, including the chloro-*s*-triazine atrazine, the chloroacetanilides metolachlor and alachlor, and the diphenylether fluorodifen. GST activities toward these substrates have then been determined in seedlings in maize and associated weeds, together with the concentration of total glutathione in each species. We have then correlated these results with the observed selectivity of the herbicides and, in the case of atrazine, to the rates of metabolism of the radiolabelled herbicide in detached leaves.

2 MATERIALS AND METHODS

2.1 Plant material and growth conditions

Seeds of maize (*Zea mays* cv. Artus; Booker Seeds, UK) were washed prior to use to remove the seed dressing. Seeds of the weed species *A. theophrasti* Medic., *Digitaria sanguinalis* (L.) Scop., *Echinochloa crus-galli* (L.) Beauv., *P. miliaceum*, *Setaria faberi* Herrm., and *Sorghum bicolor* (L.) Moench were obtained from Herbiseed Ltd, Wokingham, UK. Seeds were sown in Levington multi-purpose potting compost and grown at 20°C under a 16 h photoperiod using artificial light (150 μmol m^{-2} s^{-1}). Ten days after sowing, the aerial parts of the plants were harvested by detaching at soil level and after weighing they were frozen in liquid nitrogen and stored at −80°C.

2.2 Chemicals

Analytical grade herbicides were obtained from Greyhound Chromatography Supplies, Merseyside, L43 4XK, UK. Reference glutathione conjugates of metolachlor, alachlor, and the 2-nitro-4-trifluoromethylphenyl metabolite derived from fluorodifen[2] were prepared after dissolving the herbicides in acetone (20 mg ml^{-1}) and diluting these stock solutions to 0·5 mg ml^{-1} in 0·1 M Tris HCl buffer pH 8·8 containing 10 mM reduced glutathione. After incubating at 30°C for 24 h, the reaction products were analysed by thin layer chromatography (TLC) on silica gel 60 plates containing fluorescent indicator (Merck, Darmstadt, Germany) using solvent system 1 (butan-1-ol + acetic acid + water; 4 + 1 + 1; by volume) as the developing solvent. The conjugates were also analysed by HPLC as described below. A similar procedure was used for the triazine herbicides after preparing the respective trimethylamino salts.[14] [U-*triazinyl*-^{14}C]atrazine (288·6 MBq $mmol^{-1}$) was obtained from Sigma Chemical Co. (Poole, Dorset, BH17 7NH, UK) and routinely purified prior to use by TLC using solvent system 2 (chloroform + ethanol; 9 + 1 by volume).

2.3 Extraction and assay of GST activities

Frozen plant tissue was ground to a powder with a pestle and mortar using liquid nitrogen. The powder was then thawed in 0·1 M Tris HCl (pH 7·5) containing 1 mM EDTA, 14 mM 2-mercaptoethanol and polyvinylpolypyrrolidone (75 g kg^{-1}). After straining through two layers of muslin, the homogenate was centrifuged (15 000*g*, 15 min, 4°C) and, after decanting, the supernatant was mixed (9 + 1 by volume) with protamine sulfate solution (14 mg ml^{-1}) and then re-centrifuged under identical conditions. Finally ammonium sulfate was added to the supernatant to 80% saturation and the protein pellet collected by centrifugation as above and stored at −20°C until required.

Protein pellets were taken up and desalted in 2 mM potassium phosphate buffer (pH 6·8) on Sephadex G-25 columns (Pharmacia PD 10). The protein contents were then determined using the Bio Rad dye binding reagent as recommended by the manufacturer. After adjusting to 10 mg protein ml^{-1} this preparation was used for all enzyme assays. GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) was determined spectrophotometrically using a standard procedure[15] except that the final concentration of glutathione in the assays was 0·3 mM. GST activities toward the herbicide substrates were determined by adding the enzyme extract (120 μl) to herbicide dissolved in acetone (10 mM; 10 μl),

10 mM glutathione (20 μl adjusted to pH 7·0) and either 0·1 M potassium phosphate buffer (pH 6·8; 50 μl) for the assay of triazine and chloroacetanilide substrates or 50 mM glycine–sodium hydroxide buffer (pH 9·5; 50 μl) for the assay with fluorodifen. Control incubations consisted of (a) omitting glutathione from the incubation to correct for material which might co-chromatograph with glutathione conjugates of the herbicide and (b) omitting the enzyme, or using boiled enzyme preparations, to correct for the non-enzymatic rate of glutathione conjugation. The mixture was incubated at 37°C for 60 min and the reaction was then terminated by the addition of 0·6 M hydrochloric acid (10 μl) and the mixture frozen at −20°C. The precipitated protein was removed by centrifugation (12 000*g*, 5 min) and the supernatant (50 μl) injected onto a Spherisorb octadecyl HPLC column (250 × 4·6 mm, 5 μm particle size, Fisons Chromatography, Loughborough, Leicestershire, LE11 0RH, UK) equilibrated with solvent A + solvent B (95 + 5 by volume), where solvent A was water + phosphoric acid (99 + 1 by volume) and solvent B was acetonitrile. The column was then eluted at 0·8 ml min^{-1} with a two-step linear gradient from solvent A + solvent B (95 + 5 by volume) at time 0 to solvent A + solvent B (90 + 10 by volume) at 5 min and then to solvent A + solvent B (43 + 57 by volume) at 27 min. The eluant was monitored for UV absorbance at 264 nm and all the glutathione conjugates eluted before the end of this gradient. The column was then washed with acetonitrile for 6 min to remove unreacted herbicide prior to re-equilibrating the system. Glutathione conjugates of the herbicides were identified by co-chromatography with the synthesised reference standards and quantified following integration of UV-absorbing peaks by calibrating the system with known amounts of the corresponding herbicides. The GST-dependent formation of the conjugates was determined after correcting for the non-enzymic rate and activity was expressed as pmols of product formed s^{-1} mg^{-1} protein (pkats mg^{-1}).

2.4 Determination of glutathione content

Samples of foliage, frozen at −80°C after weighing, were ground with ice-cold trichloroacetic acid (220 mg ml^{-1}; 3 ml g^{-1} foliage) using a pestle and mortar. The homogenate was centrifuged (12 000*g*, 5 min) and the supernatant was assayed for total glutathione by the glutathione reductase-coupled assay.[16] Glutathione content was then determined by reference to a calibration curve prepared with the authentic thiol.

2.5 Metabolism studies with [^{14}C]atrazine

Leaves from 10-day-old seedlings were detached, weighed, and the cut stems of individual leaves immersed each in an aqueous solution of [^{14}C-*triazinyl*] atrazine (68 μM, 288·6 MBq $mmol^{-1}$; 1 ml) and triplicate samples incubated in the dark in the air stream from a fume cupboard for 16 h. The leaves were then removed from the feeding solutions, rinsed in distilled water and then homogenised in methanol (4 ml g^{-1} leaf) using a pestle and mortar. The extract was filtered through Whatman No. 1 filter paper, and assayed by liquid scintillation counting; the insoluble residue was assayed for radioactivity using a Packard Sample Oxidiser. The solvent extract was then concentrated to dryness under a stream of nitrogen, the residue dissolved in methanol (40–200 μl) and the solution (20 μl) applied to a TLC plate and developed in solvent system 2. Radioactive metabolites were located by autoradiography using X-ray film (Fuji Photo Film Co., Japan) and quantified by scraping the silica from radioactive areas into a scintillation vial containing methanol (0·5 ml) prior to the addition of scintillant and radioassay by scintillation counting.[15] Using solvent system 2, the herbicide and its non-conjugated metabolites, including the *N*-dealkylated derivatives, could be clearly resolved.[15] However, the polar glutathione conjugate of atrazine and related metabolites remained on the origin. To confirm their identities, the extracts were also analysed by TLC in solvent system 1 and the identities of the glutathione conjugate of atrazine and its related metabolites confirmed by co-chromatography.[15]

2.6 Herbicide treatment of plants

Seeds were sown in a sandy loam and the soil treated by spray application of herbicides using a laboratory sprayer equipped with a T-jet nozzle to deliver at a rate equivalent to 290 litres ha^{-1}. Atrazine, alachlor and metolachlor were applied as formulated products made up in water ('Atraflow', 'Lasso', and 'Dual 8E' respectively). Technical grade fluorodifen was sprayed as an acetone solution. Plants were maintained in a greenhouse with a minimum temperature of 21°C, equipped with supplementary sodium lighting (14 h photoperiod) and watered by automatic sub-irrigation. Herbicide injury was assessed visually at seven and 14 days by comparison with plants treated with water or solvent alone.

3 RESULTS

3.1 HPLC assay for the determination of multiple GST activities with herbicide substrates

A simple HPLC-based method suitable for the assay of GST activities toward a wide range of herbicides was established by resolving and quantifying the respective

glutathione conjugates. Typical elution profiles obtained for each of the herbicides incubated with a maize protein extract in the presence of glutathione are shown in Fig. 1. Using the HPLC system described above, the chemically synthesised glutathione conjugates (GS-) of the herbicides typically eluted with the retention times given in Table 1. Using the assay conditions described, the typical non-enzymic conjugation rates determined for the herbicides are also given in Table 1. The non-enzymic rates were determined each time an assay was performed and were subtracted from the reaction rate in the presence of the enzyme preparation to give the corrected specific GST activity per unit protein. Using preparations from maize to validate the system, it was demonstrated that the formation of glutathione conjugates was strictly dependent upon time over the 60-min incubation period, and on protein content, between 0 and 1·5 mg protein per assay, with either metolachlor or atrazine as substrates. The glutathione conjugates of atrazine, alachlor and the fluorodifen cleavage product were completely stable at room temperature for up to 6 h, though to avoid the risk of cleavage of the acid-labile glutamyl bond of the peptide, further storage of the samples, other than at −20°C, was avoided. To reduce the degradation of the glutathione conjugate, the lowest concentration of hydrochloric acid compatible with precipitating the proteins at the conclusion of the assay was used. Using the maize extracts, after correcting for the non-enzymic rate, the GST activities toward the chloro-*s*-triazines cyanazine and simazine were negligible and these herbicides were not used in any of the further studies.

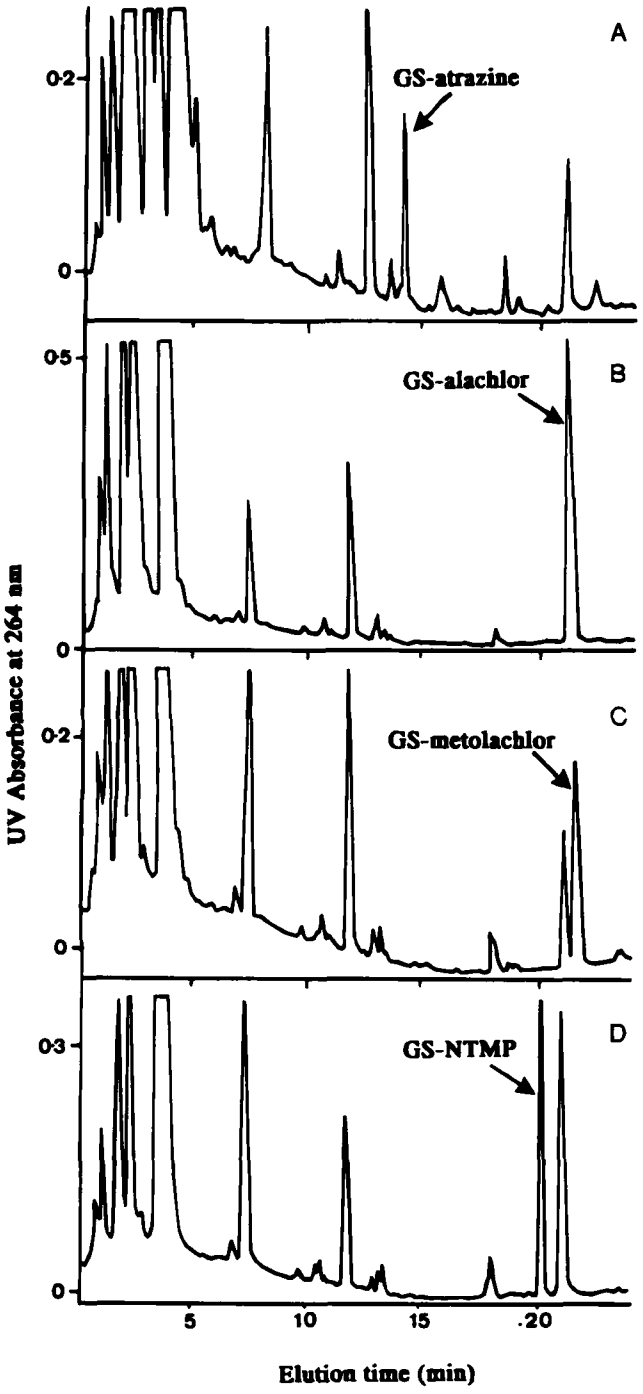

Fig. 1. HPLC chromatograms of GST reaction products formed when the herbicides atrazine (A), alachlor (B), metolachlor (C) and fluorodifen (D) were incubated with a crude desalted protein extract from maize in the presence of glutathione. The respective glutathione conjugates (GS-) are identified in each case. With fluorodifen as substrate the conjugate was the glutathione derivative of 2-nitro-4-trifluoromethylphenol (GS-NTMP) formed following the cleavage of the herbicide.

GST activities toward the non-herbicide substrate CDNB and the herbicides alachlor, metolachlor, atrazine and fluorodifen were determined in extracts from the foliage of 10-day-old seedlings of maize and associated weed species (Table 2). Comparisons of the catalytic rates with the various herbicides can be considered valid as, in contrast to previous studies where assays have been carried out under a variety of conditions with radiolabelled herbicides at relatively low concentrations, these HPLC assays have used substrate concentrations in excess of the reported K_m values for all substrates.[15,17]

TABLE 1

HPLC Retention Times of GS-conjugates and Non-enzymic Conjugation Rates for Some Herbicides

	Retention time (min)	*Non-enzymic conjugation rate (±SD)[a] pkats[b]*
Atrazine	14·2	0·062 (±0·038)
Cyanzine	14·3	nd
Simazine	12·4	nd
Alachlor	21·0	0·135 (±0·046)
Metolachlor	21·2	0·034 (±0·012)
NTMP[c]	20·0	nd
Fluorodifen	nd	0·006 (±0·002)

[a] $n = 8$.

[b] pmoles of product formed per second.

[c] 2-Nitro-4-trifluoromethyl phenol, derived by cleavage of fluorodifen.

TABLE 2
GST Activities toward CDNB[a] and Herbicide Substrates in Desalted Extracts of Foliage from 10-Day-Old Seedlings of Maize and Associated Weeds

Plant species	*Enzyme activity (±SD) pkats mg⁻¹ protein*[bc]				
	CDNB[a]	*Alachlor*	*Metolachlor*	*Atrazine*	*Fluorodifen*
Zea mays	383·50 (7·50)	0·92 (0·04)	0·45 (0·05)	1·03 (0·05)	0·45 (0·06)
Abutilon theophrasti	110·00 (0·00)	1·26 (0·13)	1·27 (0·09)	0·21 (0·01)	1·56 (0·15)
Digitaria sanguinalis	40·00 (0·00)	0·08 (0·03)	0·10 (0·00)	0·22 (0·01)	0·06 (0·00)
Echinochloa crus-galli	60·00 (0·00)	0·22 (0·04)	0·10 (0·04)	0·09 (0·01)	0·19 (0·01)
Panicum miliaceum	57·00 (2·00)	0·20 (0·01)	0·14 (0·03)	0·71 (0·08)	0·09 (0·01)
Setaria faberi	61·00 (0·00)	0·06 (0·02)	0·12 (0·02)	0·23 (0·02)	0·15 (0·01)
Sorghum bicolor	305·50 (3·50)	1·25 (0·20)	0·24 (0·05)	0·25 (0·03)	0·32 (0·02)

[a] 1-Chloro-2,4-dinitrobenzene.
[b] Values for herbicides refer to the means of triplicate determinations.
[c] With CDNB as substrate, the mean of duplicate determinations is given with the variation between the mean and the replicates given in parentheses.

3.2 Glutathione contents of seedlings

The total glutathione content in the foliage of the 10-day-old seedlings was determined using a specific glutathione reductase-coupled assay (Table 3). The accuracy of the method was confirmed by spiking plant samples with authentic glutathione prior to extraction, and the recoveries were shown to exceed 94%. Glutathione concentrations of 120–160 μM were found in all species, except *D. sanguinalis* and *S. bicolor*, which contained approximately half this amount.

3.3 Metabolism of [^{14}C]atrazine in detached leaves from seedlings

After feeding with radiolabelled atrazine for 16 h, the total extractable radioactivity in the detached leaves, together with the relative proportions of unchanged [^{14}C]atrazine, GS-atrazine and alternative metabolites were determined following TLC. In all species, combustion analysis demonstrated that a negligible proportion of the dose had become associated with the insoluble matrix from the leaf during this short feeding study. For each species, the extractable radioactivity in the leaf extracts which was not accounted for by atrazine and the glutathione conjugate, was identified as metabolites which from their chromatographic behaviour in solvent system 2 appeared to be the *N*-dealkylated derivatives of atrazine and hydroxyatrazine.[15] Similarly, these metabolites have been reported in several metabolism studies with [^{14}C]atrazine in crop and weed species[18] and their nature was not investigated further. The polar fraction remaining on the origin in solvent system 2 consisted predominantly (>70%) of authentic GS-atrazine. The remaining polar radioactive metabolites appeared to be identical to the degradation products of GS-atrazine previously determined in maize and *S. faberi*.[19] For quantification purposes the amounts of glutathione conjugate and its catabolites were added together to give a total value for the content of GS-atrazine.

TABLE 3
Total Glutathione Content in Foliage of 10-Day-Old Seedlings of Maize and Associated Weed Species

Plant species	*nmol g⁻¹ fresh weight*[a] (±*SD*)
Zea mays	143 (±19)
Abutilon theophrasti	154 (±21)
Digitaria sanguinalis	56 (± 8)
Echinochloa crus-galli	122 (±4)
Panicum miliaceum	117 (±18)
Setaria faberi	156 (±6)
Sorghum bicolor	76 (±20)

[a] Values are the mean of four determinations.

With the exception of *D. sanguinalis* and *E. crus-galli*, glutathione conjugation was the major route of herbicide metabolism in all plants (Table 4). On the basis of the percentage of the administered dose detoxified by glutathione conjugation, the efficiencies of the various plants were in the order of *A. theophrasti* > *Z. mays* > *S. bicolor* > *P. miliaceum* > *S. faberi* > *D. sanguinalis* > *E. crus-galli*. However, due to the species-dependent differences in the relative sizes of the leaves used in the experiment, a different ranking could be assigned from the relative nmols of GS-atrazine accumulating g^{-1} fresh weight. As an alternative method of representing the relative capacities of the plants to detoxify atrazine by glutathione conjugation, the ratio of unchanged atrazine to GS-atrazine was calculated for each species. Assuming that all of the atrazine present would be available for glutathione conjugation, representing the data in this way should allow a more accurate interpretation of the relative conjugating capacities, with the plants with the lowest ratios being

TABLE 4
Uptake and Metabolism of [^{14}C-*triazinyl*]Atrazine in Detached Leaves from 10-Day-Old Seedlings of Maize and Associated Weeds

Plant species	[^{14}C] *Taken up* (%)[a] (±*SD*)[b]				[*GS-AZ*[c]] (*nmol* g^{-1})
	Total	*Unchanged atrazine*	*GS-AZ*[c]	*Other metabolites*	
Zea mays	23·6 (±8·2)	5·7 (±1·3)	12·4 (±6·0)	5·5 (±5·0)	0·92 (±0·19)
Abutilon theophrasti	40·7 (±18·5)	13·0 (±1·6)	17·5 (±6·13)	0·27 (±0·02)	20·76 (±11·08)
Digitaria sanguinalis	4·2 (±0·9)	1·4 (±1·1)	1·0 (±0·7)	1·8 (±1·0)	4·96 (±1·31)
Echinochloa crus-galli	7·2 (±3·9)	5·7 (±3·8)	0·6 (±0·2)	0·9 (±0·2)	0·96 (±0·28)
Panicum miliaceum	6·8 (±1·1)	3·0 (±0·4)	3·5 (±0·1)	0·3 (±0·1)	5·55 (±1·42)
Setaria faberi	5·7 (±0·5)	3·5 (±1·1)	1·9 (±0·7)	0·3 (±0·1)	2·52 (±1·44)
Sorghum bicolor	13·2 (±4·9)	14·0 (±9·9)	5·7 (±3·4)	0·8 (±0·6)	0·40 (±0·09)

[a] Percentage of the dose administered.
[b] Values refer to means of triplicate determinations.
[c] GS-AZ = Glutathione conjugate of atrazine.

the most efficient. When calculated this way the ranking was in the order *Z. mays* (ratio = 0·50) > *A. theophrasti* (0·7) > *P. miliaceum* (0·9) > *D. sanguinalis* (1·4) > *S. faberi* (1·8) > *S. bicolor* (2·5) > *E. crus-galli* (9·5).

3.4 Herbicide selectivity studies

Although the selectivity of the herbicides used in this study has been well established, it was of interest to determine how the selectivity of atrazine, metolachlor, alachlor and fluorodifen related to the relative rates of detoxification by glutathione conjugation in the seedlings used in this study. Herbicides were applied pre-emergence and the injury to the seedlings assessed at seven and 14 days (Table 5). Although multiple application rates and post-emergence applications were applied, the data presented were found to be most representative for a comparative study with young seedlings. All the herbicides tested showed selectivity toward maize when applied pre-emergence. Subsequent post-emergence applications confirmed a similar selectivity for atrazine, metolachlor and alachlor, but the limited selectivity of fluorodifen, which is not considered a selective herbicide for use in maize, was lost. In addition to maize, other notable cases of differential tolerance to the herbicides were determined. *S. bicolor* was tolerant to pre-emergence applications of atrazine and the chloroacetanilides, especially metolachlor. *A. theophrasti* was susceptible to atrazine but tolerant of both chloroacetanilides and fluorodifen, and *P. miliaceum* was resistant to atrazine but susceptible to the other herbicides.

4 DISCUSSION

In this study we have characterised the glutathione-dependent herbicide-detoxification system in a major

TABLE 5
Herbicide Selectivity in Seedlings of Maize and Associated Weeds following Pre-emergence Herbicide Application

Plant species	*Injury* (%)[a]							
	Atrazine[b]		*Metolachlor*[c]		*Alachlor*[c]		*Fluorodifen*[c]	
	7 d	*14 d*	*7 d*	*14 d*	*7 d*	*14 d*	*7 d*	*14 d*
Zea mays	0	0	10	10	0	20	20	20
Abutilon theophrasti	50	100	0	0	0	10	10	10
Digitaria sanguinalis	40	40	70	90	70	70	70	60
Echinochloa crus-galli	50	60	80	90	95	90	80	60
Panicum miliaceum	0	0	95	50	100	50	100	100
Setaria faberi	10	70	90	90	40	50	80	60
Sorghum bicolor	5	10	0	0	20	20	50	50

[a] Values refer to relative levels of herbicide injury as compared with controls. 100% = plant death, 0–100% = percentage reduction in plant height.
[b] Application rate equivalent to 250 g AI ha^{-1}.
[c] Application rate equivalent to 125 g AI ha^{-1}.

crop species, maize, and in associated weeds, by determining GST activities toward herbicide substrates and the availability of glutathione, using uniform experimental procedures. Although the GSTs of maize have been better studied than in any other plant there have been only a handful of reports in which GST activities with more than one herbicide substrate in this species have been measured.[15,20,21] When determined under conditions of saturating substrate concentrations, maize seedlings contain GST activities toward the differing classes of substrates in the order alachlor > fluorodifen > atrazine > metolachlor. In the case of the chloroacetanilides, the results with alachlor and metolachlor confirm earlier reports showing that, as compared with metolachlor, alachlor was a preferred substrate for glutathione conjugation in maize both *in vivo* and *in vitro*.[20] In the weed species, a similar order of activities toward the herbicides was obtained to that determined in maize, with the exception of *A. theophrasti*, where the activity toward fluorodifen was marginally higher than that toward alachlor. Many of the weed species contained a surprisingly varied range of GST activities, with all species showing measurable activities toward all of the herbicide substrates. In all cases, none of the GST activities toward the herbicide substrates correlated with the activities toward the colorimetric substrate CDNB, which was the optimal substrate in all species. This observation reinforces previous comments regarding the limited usefulness of this substrate when attempting to predict GST activities toward herbicides in plant metabolism studies.[15,22] With two notable exceptions, the GST activities in seedlings of maize toward all herbicides were higher than those determined in the weed species. The exceptions were *A. theophrasti*, which had the highest GST activities of any plant toward alachlor, metolachlor and fluorodifen, and *S. bicolor*, which contained higher activities toward alachlor.

By relating GST activities to glutathione availability and relative herbicide tolerance and, in the case of atrazine, to metabolism *in vivo*, several conclusions may be drawn regarding the role of GST-mediated detoxification in herbicide selectivity in seedlings.

With atrazine as substrate, the specific activities of the GSTs were determined in the order *Z. mays* > *P. miliaceum* ≫ *S. bicolor* = *S. faberi* = *D. sanguinalis* = *A. theophrasti* > *E. crus-galli*. Significantly, as determined from the ratio of atrazine to GS-atrazine, a similar order was observed in the relative capacities of the grass species to detoxify [^{14}C]atrazine by glutathione conjugation *in vivo*. However, an exception to this correlation was observed with *A. theophrasti*, which, although susceptible to atrazine, was the most efficient species in taking up and conjugating atrazine *in planta* despite having one of the lowest extractable levels of the corresponding GST activity. Presumably, *A. theophrasti* conjugated more of the herbicide than the grass species with similar GST activities, due to the greater availability of atrazine in its tissues. Alternatively, the actual GST activities toward atrazine in this species have been underestimated, due to the reported instability of the extractable GST enzyme,[11] even though suitable precautions were taken and the extracts showed high activities toward other GST substrates. In either event, such an aberration suggests that simple correlations between enzyme activities *in vitro* and detoxification rates *in vivo* may only be established for similar groups of plants, for example the grasses. When comparing diverse species, factors such as the relative rates of uptake and translocation may become more significant. With respect to the herbicide selectivity data, it was significant that the two species with the highest GST activities toward atrazine, maize and *P. miliaceum*, were tolerant to the herbicide. However, *S. bicolor*, which was only slightly injured by atrazine, contained much lower levels of the GST, though the metabolism studies with [^{14}C]atrazine did suggest that this species efficiently absorbed and conjugated the herbicide *in vivo*. Significantly, GSTs with comparable activities in conjugating atrazine have previously been described in maize and *P. miliaceum*.[12] GST activities toward this herbicide have also been determined in *S. faberi*[13,19,23] and *A. theophrasti*.[11] However, this enzyme remains largely uncharacterised at the biochemical and molecular level. Since resistance to atrazine due to enhanced GST expression has already been reported in a biotype of *A. theophrasti*,[11] the presence of this activity in all the weed species tested suggests that a similar mechanism of resistance could evolve in other species.

A good correlation could be established in the various species between the relative GST-specific activities toward alachlor and herbicide tolerance. Maize, *A. theophrasti* and *S. bicolor* all contained GSTs highly active in detoxifying alachlor and showed minimal herbicide injury, while the other weed species showed four- to five-fold lower enzyme activities and were susceptible. In non-safener-treated maize, the detoxification of alachlor is associated with the activity of the major constitutive isoenzymes GST I and GST III.[5,6,20] However, it has also been reported that rapid conjugation of this herbicide can occur in the absence of any GST, suggesting that the availability of glutathione may also regulate relative rates of detoxification and hence herbicide selectivity.[24] Our results would support this proposal. Thus, *S. bicolor* contained higher GST activities toward alachlor than did maize, but contained less glutathione. Significantly *S. bicolor* showed some injury when treated with alachlor, while maize was unaffected. Similarly, alachlor was less damaging to *S. faberi* than to *D. sanguinalis*, *E. crus-galli* and *P. miliaceum*, and *S. faberi* contained higher concentrations of glutathione than the other plants but comparable GST activities. It was also apparent that there was a relationship between the relative GST activities toward alachlor and metolachlor in

the various species. This might be expected as the same GST isoenzymes are reported to catalyse the conjugation of both herbicides in maize, with alachlor being the preferred substrate.[20] Our results would suggest that this is likely to be the case in other species, although it was of interest to note that, while the ratios of activity toward alachlor and metolachlor were between 2:1 and 1:1 in the majority of plants, in *S. bicolor* the ratio was 5:1. With alachlor we were, therefore, able to establish a connection between glutathione-mediated detoxification *in vitro* and herbicide selectivity. Similarly, the selectivity of the related chloroacetanilide acetochlor in a range of grass and broadleaf species was correlated with the relative rates of glutathione conjugation *in vivo*.[25]

With metolachlor an excellent correlation could be established between the relative GST activities with this substrate and herbicide selectivity. *A. theophrasti*, maize and *S. bicolor* all contained high GST activities toward metolachlor and were tolerant, while the other weeds contained lower activities and were susceptible. Constitutively expressed GSTs catalysing the detoxification of metolachlor have been identified in maize (GSTs I and III)[5,17,20] and sorghum,[10] although the GST in *A. theophrasti*, which was the most active of all the GSTs assayed with this substrate, has not been reported previously. In a recent report, a GST preparation from *S. faberi* showed no activity towards metolachlor.[13] However, our results would suggest that the GSTs of *S. faberi* do show detectable activity toward metolachlor, comparable to that determined in other grass weeds.

Fluorodifen is not considered to be a selective herbicide for use in maize, but was included in this study as the GSTs catalysing its detoxification represent a widely distributed class of enzymes distinct from the other GST activities.[22] All of the species tested showed signs of phytotoxicity when treated with fluorodifen, with *A. theophrasti* being the most tolerant. Significantly, this species also contained considerably higher levels of the detoxifying GST activity than the other species. Maize showed relatively minor damage when treated with fluorodifen and contained significantly higher GST activities than many of the susceptible weeds. All of the other species were susceptible to fluorodifen and, with the exception of *S. bicolor*, contained similar low GST activities towards the herbicide.

These results suggest that herbicide selectivity can be predicted in crop and weed species from relative GST activities with a variety of herbicide substrates. In addition, studies with radiolabelled atrazine confirmed that, among the grass species, relative GST activities reflected the rates of glutathione conjugation *in vivo*, confirming the link between these enzymes and relative rates of detoxification and sensitivity to herbicides. In contrast, no simple relationship could be established between glutathione content in the different species and herbicide selectivity, with the possible exception of alachlor. As glutathione can exist in plants in both its oxidised and reduced forms it could be argued that the determination of total glutathione[16] may give no indication of the amount of reduced glutathione available for herbicide detoxification. However, a review of the literature would suggest that, under normal conditions, the majority of the glutathione pool in plants is in the reduced form.[26] Presumably, a direct relationship between glutathione availability and herbicide selectivity can be established only with highly electrophilic substrates which do not require a GST to catalyse their conjugation.

Establishing a simple relationship between relative GST activities and herbicide selectivity has a number of limitations. In the case of atrazine, although a relationship between selectivity and glutathione conjugation *in vivo* and *in vitro* could be inferred among the grass species, this relationship did not extend to the broadleaf weed *A. theophrasti*. The metabolism study in detached leaves also suggested that the contribution made by alternative routes of detoxification, together with relative rates of uptake and translocation, should not be ignored. Similarly, in various accessions of *Setaria* it was concluded that GST activities could not account for the variations in tolerance to atrazine and metolachlor and the involvement of a variety of factors such as relative rates of plant growth was implicated.[13] Even if GSTs were the sole determinants of selectivity, measuring activities *in vitro* neglects factors which are known to regulate the activity of enzymes in plants *in vivo*. In the case of GSTs, such controlling factors include the relative distribution and compartmentalisation of the enzyme and the herbicide, the availability of reduced glutathione and the kinetic efficiency of the enzyme at varying substrate concentrations. In addition, the glutathione conjugates of the herbicides are potent competitive inhibitors of GSTs[22] and the relative activities of the vacuolar transporters of the conjugates[27] together with the peptidases involved in conjugate turnover[2] may ultimately have a regulatory role *in vivo*.

ACKNOWLEDGEMENTS

Pamela Hatton and David Dixon acknowledge joint support for their CASE studentships from the Biotechnology and Biological Sciences Research Council and Rhône-Poulenc Agriculture Ltd. The authors thank Nicola Slack for her assessments of herbicide selectivity.

REFERENCES

1. Cole, D. J., Detoxification and activation of agrochemicals in plants. *Pestic. Sci.*, **42** (1994) 209–22.
2. Lamoureux, G. L., Shimabukuro, R. H. & Frear, D. S., Glutathione and glucoside conjugation in herbicide selectivity. In *Herbicide Resistance in Weeds and Crops*, ed.

J. C. Caseley, G. W. Cussans & R. K. Aitkin. Butterworth-Heinemann, Oxford, 1991, pp. 227–61.
3. Timmermann, K. P., Molecular characterisation of corn glutathione *S*-transferase isozymes involved in herbicide detoxication. *Physiol. Planta*, **77** (1989) 465–71.
4. Shimabukuro, R. H., Frear, D. S., Swanson, H. R. & Walsh, W. C., Glutathione conjugation: an enzymatic basis for atrazine resistance in corn. *Plant Physiol.*, **47** (1971) 10–14.
5. Irzyk, G. P. & Fuerst, E. P., Purification and characterisation of a glutathione *S*-transferase from benoxacor-treated maize (*Zea mays*). *Plant Physiol.*, **102** (1993) 803–10.
6. Jepson, I., Lay, V. J., Holt, D. C., Bright, S. W. J. & Greenland, A. J., Cloning and characterisation of maize herbicide safener-induced cDNAs encoding subunits of glutathione *S*-transferase isoforms I, II and IV. *Plant Mol. Biol.*, **26** (1994) 1855–66.
7. Lamoureux, G. L. & Rusness, D. G., EPTC metabolism in corn, cotton, and soybean: identification of a novel metabolite derived from the metabolism of a glutathione conjugate. *J. Agric. Food Chem.*, **35** (1987) 1–7.
8. Diesperger, H. & Sandermann, H., Soluble and microsomal glutathione *S*-transferase activities in pea seedlings (*Pisum sativum* L.). *Planta*, **146** (1979) 643–8.
9. Schroder, P. & Berkau, C., Characterisation of cytosolic glutathione *S*-transferase in spruce needles. Part 1: GST isoenzymes of healthy trees. *Bot. Acta*, **106** (1993) 301–6.
10. Dean, J. V., Gronwald, J. W. & Eberlein, C. V., Induction of glutathione *S*-transferase isoenzymes in sorghum by herbicide antidotes. *Plant Physiol.*, **92** (1990) 467–73.
11. Anderson, M. P. & Gronwald, J. W., Atrazine resistance in a velvetleaf (*Abutilon theophrasti*) biotype due to enhanced glutathione *S*-transferase activity. *Plant Physiol.*, **96** (1991) 104–9.
12. Ezra, G. & Stephenson, G. R., Comparative metabolism of atrazine and EPTC in proso millet (*Panicum miliaceum* L.) and corn. *Pestic. Biochem. Physiol.*, **24** (1985) 207–12.
13. Wang, R-L. & Dekker, J., Weedy adaptation in *Setaria* spp. III. Variation in herbicide resistance in *Setaria* spp. *Pestic. Biochem. Physiol.*, **51** (1995) 99–116.
14. Crayford, J. V. & Hutson, D. H., The metabolism of the herbicide, 2-chloro-4-(ethylamino)-6-(1-cyano-methyl-ethylamino)-*s*-triazine in the rat. *Pestic. Biochem. Physiol.*, **2** (1972) 295–307.
15. Edwards, R. & Owen, W. J., Comparison of glutathione *S*-transferases of *Zea mays* responsible for herbicide detoxification in plants and suspension cultured cells. *Planta* **169** (1986) 208–15.
16. Anderson, M. E., Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol.*, **113** (1985) 548–55.
17. Mozer, T. J., Tiemeier, D. C. & Jaworski, E. G., Purification and characterisation of corn glutathione *S*-transferase. *Biochem.*, **22** (1983) 1068–72.
18. Cole, D. J., Edwards, R. & Owen, W. J., The role of metabolism in herbicide selectivity. In *Herbicides*, ed. D. H. Hutson & T. R. Roberts. John Wiley and Sons, Chichester, 1987, pp. 57–103.
19. Hatton, P. J., Edwards, R. & Cole, D. J., Glutathione transferases in major weed species. *Pestic. Sci.*, **43** (1995) 173–5.
20. O'Connell, K. M., Breaux, E. J. & Fraley, R. T., Different rates of metabolism of two chloroacetanilide herbicides in Pioneer 3320 corn. *Plant Physiol.*, **86** (1988) 359–63.
21. Dean, J. V., Gronwald, J. W. & Anderson, M. P., Glutathione *S*-transferase activity in non-treated and CGA-154281-treated maize shoots. *Z. Naturforsch.*, **46c** (1991) 850–6.
22. Lamoureux, G. L. & Rusness, D. G., Glutathione in the metabolism and detoxification of xenobiotics in plants. In *Sulfur Nutrition and Assimilation in Higher Plants*, ed. I. J. De Kok, I. Stulen, H. Rennenberg, C. Brunold & W. Rauser. SPR Academic Publishing, The Hague, Netherlands, 1993, pp. 221–37.
23. Lamoureux, G. L. & Rusness, D. G., Tridiphane [2-(3,5-dichlorophenyl)-2-(2,2,2-trichloroethyl)oxirane] an atrazine synergist: enzymatic conversion to a potent glutathione *S*-transferase inhibitor. *Pestic. Biochem. Physiol.*, **26** (1986) 323–42.
24. Jablonkai, I. & Hatzios, K. K., *In-vitro* conjugation of chloroacetanilide herbicides and atrazine with thiols and contribution of nonenzymatic conjugation to their glutathione-mediated metabolism in corn. *J. Agric. Food Chem.*, **41** (1993) 1736–42.
25. Breaux, E. J., Initial metabolism of acetochlor in tolerant and susceptible seedlings. *Weed Sci.*, **35** (1987) 463–8.
26. Alscher, R. G., Biosynthesis and antioxidant function of glutathione in plants. *Physiol. Planta*, **77** (1989) 457–64.
27. Gaillard, C., Dufaud, A., Tommasini, R., Kreuz, K., Amrhein, N. & Martinoia, E., A herbicide antidote (safener) induces the activity of both the herbicide detoxifying enzyme and of a vacuolar transporter for the detoxified herbicide. *FEBS. Letts*, **352** (1994) 219–21.